Effect of Initial Slight Oxidation on Stability of Polyunsaturated Fatty Acid/Protein Mixtures Under Controlled Atmospheres

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ABSTRACT: A mixture of unsaturated fatty acids (FA), mainly linolenic acid, and bovine serum albumin (BSA) was incubated under several different atmospheres to study the effect of these atmospheres on the stability of FA to oxidation. Four experiments were carried out simultaneously, which consisted of the incubation of the FA/BSA mixture under air, nitrogen, air in the presence of 200 ppm of butylated hydroxytoluene, and air for 6 h and then under nitrogen. The four experiments were tested for lipid oxidation and color changes by measuring absorbances at 234 and 270 nm, formation of thiobarbituric acid-reactive substances, and color differences and yellowness index. The samples that were oxidized with air before storage under nitrogen were the most stable against lipid peroxidation and exhibited the smallest color changes. These results suggest that a slight and controlled lipid oxidation improved the oxidative stability of FA/BSA mixtures.
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KEY WORDS: Amino acid modification, antioxidative activity, controlled atmospheres, lipid peroxidation, nonenzymatic browning, oxidized lipid/protein reactions.

Lipid oxidation is one of the major causes of food spoilage and is undesirable not only from an acceptability and economic point of view but also because oxidative reactions can decrease the nutritional quality of food and generate oxidation products that are potentially toxic (1–3). Because it is a great economic concern to the food industry, extensive research has been done not only to identify the products of lipid oxidation and the conditions that influence their production but also to study the mechanisms involved (4–6). Since oxidative reactions in food lipids are exceedingly complex, simpler model systems, such as oleate, linoleate and linolenate, have been used to ascertain mechanistic pathways (7–9). However, other components present in foods are able to play a role in the lipid peroxidation process, which can be either accelerated or delayed with respect to the oxidation of pure lipids.

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In this context, previous studies from this laboratory have shown that the reaction of lipid oxidation products with reactive groups of amino acids and proteins produced oxidized lipid/amino acid reaction products (OLAARP) with antioxidative properties (10–12), and these products were able to delay the lipid peroxidation process at the same time that they were being produced (13,14). All these results have suggested some surprising possibilities in preservation of food. If OLAARP could be produced in food, this process might increase the oxidative stability of those foods without the addition of additives. As a continuation of those studies, the present investigation was undertaken to study the effect of a slight oxidation on the fatty acid stability of unsaturated fatty acid/bovine serum albumin (FA/BSA) mixtures stored under different atmospheres. Because OLAARP are likely produced when lipid peroxidation occurs in the presence of proteins (15), initially oxidized mixtures may contain a small amount of OLAARP that may enhance the sample stability.

EXPERIMENTAL PROCEDURES

Materials. A commercial mixture of FA containing linolenic acid (~70%), linoleic acid (~20–25%), and oleic acid (~3%) was obtained from Fluka (Buchs, Switzerland). Essentially fatty acid-free BSA, obtained by cold alcohol precipitation (≥96% BSA), and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Thiobarbituric acid monohydrate was purchased from Merck (Darmstadt, Germany). Other reagents and solvents used were analytical grade and were purchased from reliable commercial sources.

Sample preparation. A mixture of FA (23% w/w) and BSA (77% w/w) was triturated in a mortar until a homogeneous powder was obtained, and rapidly used to avoid oxidation. The FA/BSA mixture (2 g) was introduced in a 25-mL glass bottle and hermetically sealed using a polypropylene cap with polytetrafluoroethene liner. Four experiments were carried out simultaneously, which consisted of the incubation of the FA/BSA mixture under: A, air; B, nitrogen; C, air in the presence of 200 ppm of BHT; and D, air for 6 h and then nitrogen. Experiments A–C were controls to analyze the lipid per-

oxidation process in the FA/BSA model system under several atmospheres and in the presence of BHT. In experiment D, the first 6 h were used to induce a slight lipid oxidation. After this initial oxidation, the stability of the FA/BSA mixture was studied under an inert atmosphere. The four experiments were incubated for 60 d in the dark at 60°C. At different periods of time the bottles were opened, 15-mg samples were removed for analytical measurements, and, immediately, the atmospheres were replaced and the bottles sealed.

Analytical measurements. Lipid peroxidation and color changes were evaluated periodically. Color changes were determined using a Minolta CR200 chromameter (Minolta Camera Co., Osaka, Japan). The difference of color (ΔE) between incubated and initial samples was determined by the following equation (16):

$$\Delta E = \left[(\Delta a^*)^2 + (\Delta b^*)^2 + (\Delta L^*)^2\right]^{1/2} \quad [1]$$

using the CIELAB $L^*a^*b^*$ color values (17). Yellowness index (YI) was expressed according to Francis and Clydesdale (18) as:

$$\text{YI} = 142.86 \times b^*/L^* \quad [2]$$

Three determinations were used to evaluate lipid peroxidation: the absorbances at 234 and 270 nm, and the thiobarbituric acid-reactive substances (TBARS) assay. Absorbances at 234 and 270 nm were measured in the organic layer resulting from shaking 5.0 mg of the FA/BSA mixture with 25 mL of isooctane. The TBARS assay was carried out by the method of Kosugi *et al.* (19) using the solution produced from shaking 5 mg of FA/BSA mixture with 1 mL of methanol.

Statistical analysis. Color and TBARS determinations are expressed as mean values of three experiments. Statistical comparisons among several groups were made using analysis of variance (ANOVA). When significant F values were obtained, group differences were evaluated by the Student-Newman-Keuls test (20). All statistical procedures were carried out using *Primer of Biostatistics: The Program* (McGraw-Hill, Inc., New York). Significance level is $P < 0.05$ unless otherwise indicated.

RESULTS

Fatty acid oxidation in FA/BSA mixtures incubated under air. The high percentage of linolenic acid in FA made the FA/BSA mixtures very prone to oxidation. Thus, when an FA/BSA mixture was incubated under air in the dark at 60°C, lipid peroxidation occurred very rapidly. Figure 1 shows the change in absorbance at 234 and 270 nm, TBARS, and YI in an FA/BSA mixture incubated under air. All determinations have been included in the same graph for comparison purposes. Units are arbitrary and the maximum value obtained for each determination was normalized to 100.

Absorbance at 234 nm is mostly related to production of conjugated hydroperoxides, the first step in the lipid peroxidation process. Therefore, this was the first determination that exhibited changes, and the absorbance increased from 0.006 to 0.213 for the first 6 h. By using an absorptivity of 25,738 for hydroperoxides (21), and assuming that this absorbance was mostly due to fatty acid hydroperoxides, the percentage of hydroperoxides in total lipids increased from 0.1 to 5.6% in the first 6 h, and continued increasing in the next 66–90 h until a maximum of 21.4% was obtained after 72–96 h. After this time the percentage of hydroperoxides began to decrease and, after 384 h, only a residual absorbance of 0.12 was obtained. The residual absorbance might be due to the absorbance produced by secondary products of lipid peroxidation.

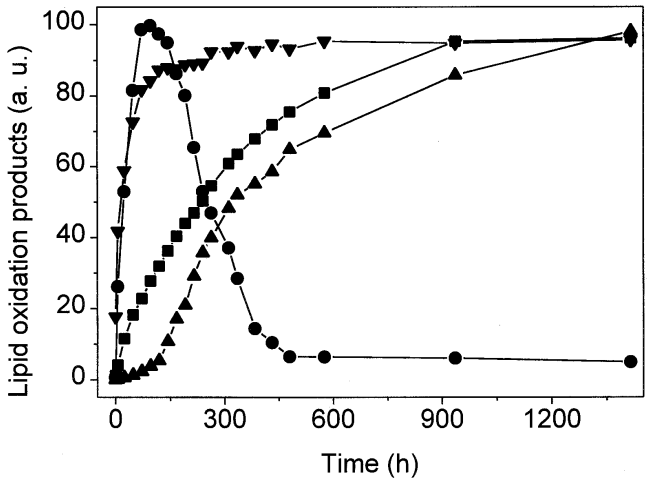

FIG. 1. Changes produced in a fatty acid/bovine serum albumin (FA/BSA) mixture incubated under air in the dark at 60°C (experiment A). Absorbances at 234 (●) and 270 (▲), thiobarbituric acid-reactive substances (TBARS) (■), and Yellowness Index (YI) (▼) were evaluated at different periods of time. Units are arbitrary and all the measurements have been included in the same graph for comparison purposes.

Formation of secondary products of lipid peroxidation was evaluated by both the absorbance at 270 nm and the TBARS assay. The absorbance at 270 nm exhibited a lag period for the first 120 h in which only slight increases in the absorbance were observed (absorbance increased from 0 to 0.026). When the percentage of hydroperoxides began to decrease after reaching its maximum, a large increase in absorbance at 270 nm was observed, probably due to the production of secondary products from hydroperoxides. These values continued increasing for the next 60 d until an absorbance of 0.471 was reached, which was followed by a slight decrease afterward. The absorbance after six months was 0.352.

The change in TBARS was similar to the change in absorbance at 270 nm, although no lag period was observed, possibly due to reaction of hydroperoxides with the thiobarbituric acid under the conditions of the assay in which hydroperoxides might be decomposed (19). This increase in TBARS was significant ($P < 0.05$) after the first 6 h and continued increasing for the first 2 mon until a maximum value of 21,000 pmol per mg of FA/BSA mixture. After 2 mon, TBARS values decreased. After 7 mon, the value was 9000 pmol per mg of FA/BSA mixture.

Color changes in the FA/BSA mixture were related to both lipid oxidation and OLAARP formation (22–24). These measurements were carried out to study the discoloring produced in the samples. Figure 1 only includes values for YI in order to simplify the figure. Nevertheless, analogous results were obtained for ΔE. Samples exhibited a very rapid increase of YI for the first 72 h, which was significant after the first 6 h, and a much slower increase of YI afterward.

Effect of treatments on hydroperoxide formation in FA/BSA mixtures. Figure 2 shows the absorbance at 234 nm of the organic extracts obtained for the four experiments carried out with the FA/BSA mixture as a function of the incubation time. As discussed previously, the incubation of the FA/BSA mixture under air (experiment A) rapidly produced hydroperoxides that exhibited the maximum percentage (21.4 %) after 72–96 h. When the mixture was stored under nitrogen (experiment B) the maximum of hydroperoxides was produced similarly but the percentage obtained was much lower (5.7%). Analogous percentages of hydroperoxides and kinetics were also obtained for the mixture incubated under air in the presence of BHT (experiment C). However, the kinetics of hydroperoxide formation observed for samples oxidized with air and then stored under nitrogen (experiment D) were slightly different. After the first 6 h the absorbance at 234 nm was analogous to samples incubated with air, but when the air was replaced with nitrogen, this absorbance only increased for a short period, then followed kinetics that were similar to samples from experiments B and C, and after 168 h, began to decrease very rapidly. After 336 h samples of experiment D had a residual absorbance of 0.04, a value that samples of experiments B or C only exhibited after 2136 and 1416 h, respectively.

Effect of treatments on secondary products formation in FA/BSA mixtures. Much greater differences than those observed for absorbances at 234 nm were obtained for absorbances at 270 nm or for TBARS. Figure 3 shows the time course of absorbance at 270 nm. The lag period observed for samples incubated in the presence of air increased for the other experiments, which exhibited much lower values of absorbance after 60 d. In addition, the kinetics were different for the different experiments. Thus, samples in experiment B showed a slower increase as a function of time than samples in experiment A. Nevertheless, after 480 h an absorbance of

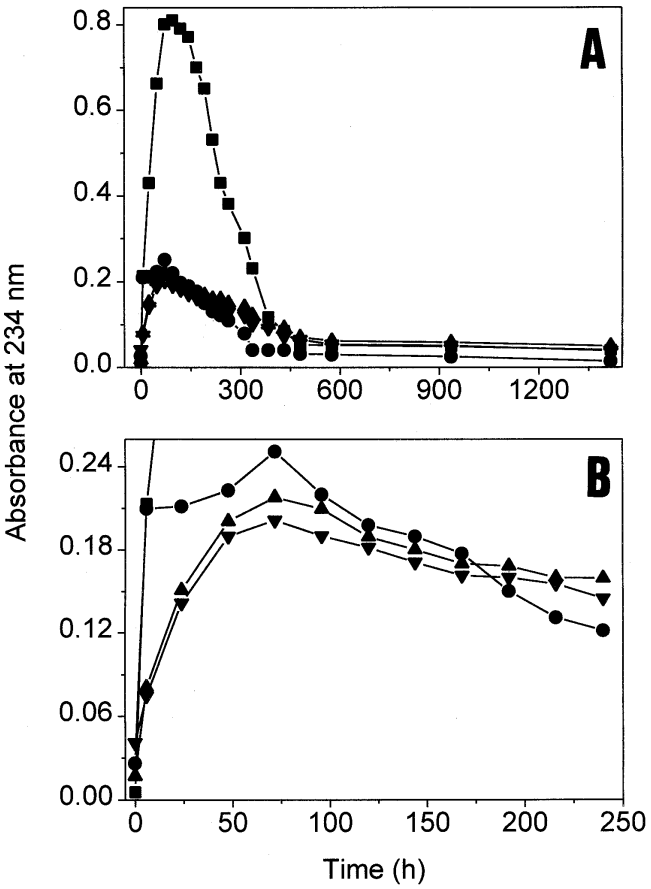

FIG. 2. Effect of the treatment on the changes produced in the absorbance at 234 nm of an FA/BSA mixture. Four experiments were carried out simultaneously, which consisted of the incubation of the FA/BSA mixture at 60°C in the dark under: A, air (■); B, nitrogen (▲); C, air in the presence of 200 ppm of butylated hydroxytoluene (▼); and D, air for the first 6 h and then under nitrogen (●). The first 250 h are expanded in part B of the figure. See Figure 1 for abbreviations.

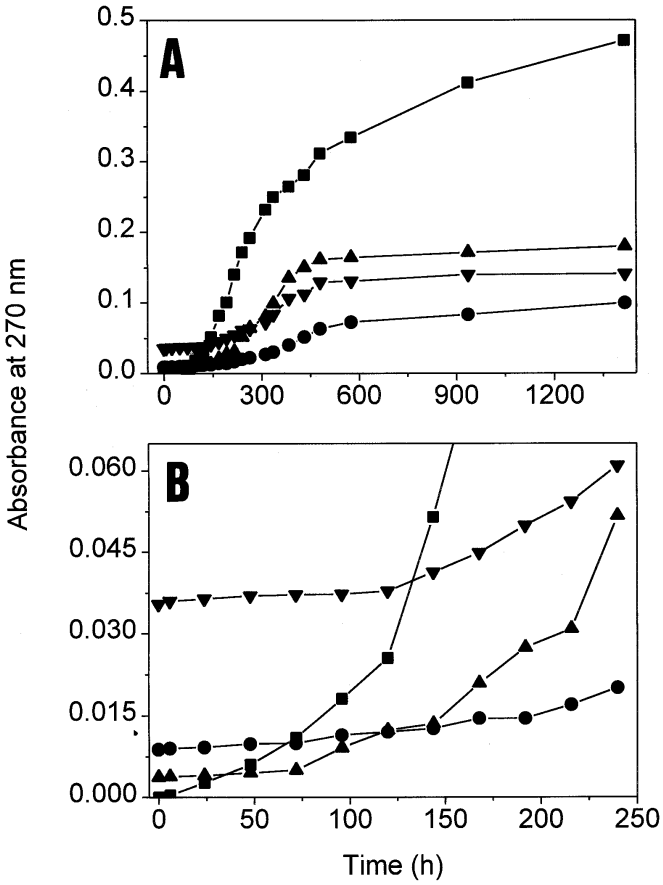

FIG. 3. Effect of the treatment on the changes produced in the absorbance at 270 nm of an FA/BSA mixture. See Figure 2 for experimental details. The first 250 h of treatment are expanded in part B of the figure. See Figure 1 for abbreviations.

0.16 was obtained that increased slowly until a maximum of 0.18 was obtained after 1416 h. The absorbance of samples treated with BHT was initially higher than other samples, more likely due to the absorption by BHT at this wavelength. However, this value increased less than values of samples in experiment B, and differences between initial and final absorbances were 0.176 for samples incubated under nitrogen, and 0.105 for samples incubated with BHT. Nevertheless, the lower increase was observed for samples in experiment D. These samples were very stable with incubation time and the difference between initial and final absorbances was 0.091.

Results analogous to those obtained for the absorbance at 270 nm were also observed for TBARS (Fig. 4), and BHT was not an interference for this last measurement. Thus, no lag period was observed and samples incubated under air significantly increased TBARS from the beginning. When the FA/BSA mixture was incubated under nitrogen, TBARS also increased with time, but the values obtained were much lower than those obtained for samples in experiment A. TBARS of samples in experiment B were significantly lower than TBARS of samples in experiment A after 6 h. BHT protected more than nitrogen and TBARS were significantly lower for samples in experiment C than for samples in experiment B after 48 h of incubation. However, the lowest TBARS values with time were obtained for samples in experiment D. After 6 h, samples in experiment D had TBARS analogous to samples in experiment A, and significantly higher than samples in experiments B or C. However, when the air was replaced with nitrogen, TBARS continued increasing very slowly. The samples in experiment D exhibited TBARS analogous to the samples in experiment B after 96 h, and TBARS in experiment D were significantly lower than TBARS in experiment B after 168 h. In addition, samples in experiment D exhibited TBARS analogous to the samples in experiment C after 168 h, and had significantly lower TBARS than samples in experiment C after 240 h.

Effect of treatments on the color of FA/BSA mixtures. No large differences in color changes were observed among the four treatments evaluated; however, these differences were significant. Figure 5 shows ΔE obtained for the four treatments as a function of time. The highest ΔE were observed for samples in experiment A, followed by samples in experiment B, and, finally, samples in experiment C. Samples in experiment D exhibited big color differences at the beginning, which were analogous to samples in experiment A and significantly higher than samples in experiments B or C, but then

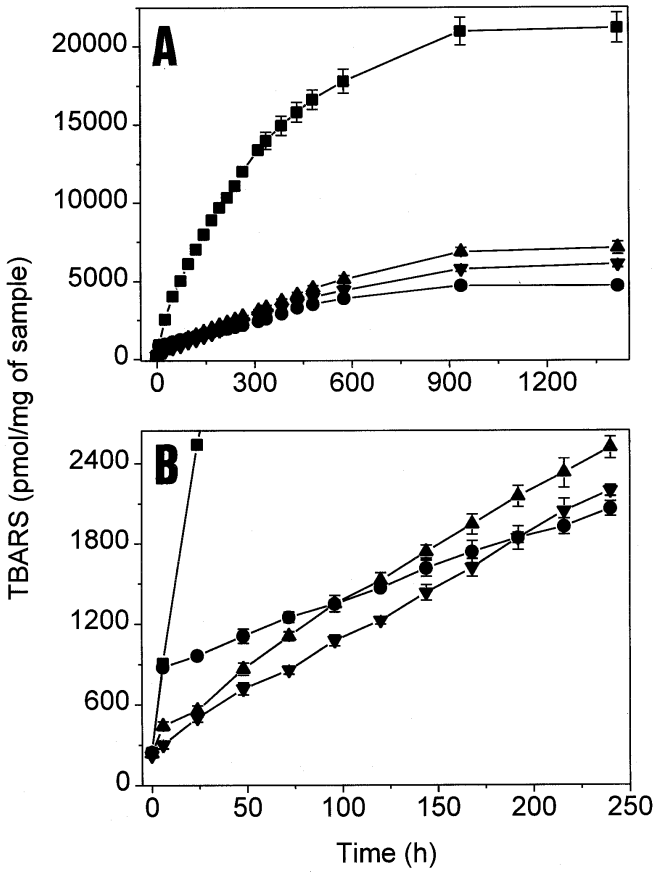

FIG. 4. Effect of the treatment on the changes produced in TBARS of a FA/BSA mixture. See Figure 2 for experimental details. Results represent the mean ± SD of three assays. The first 250 h of treatment are expanded in part B of the figure. See Figure 1 for abbreviations.

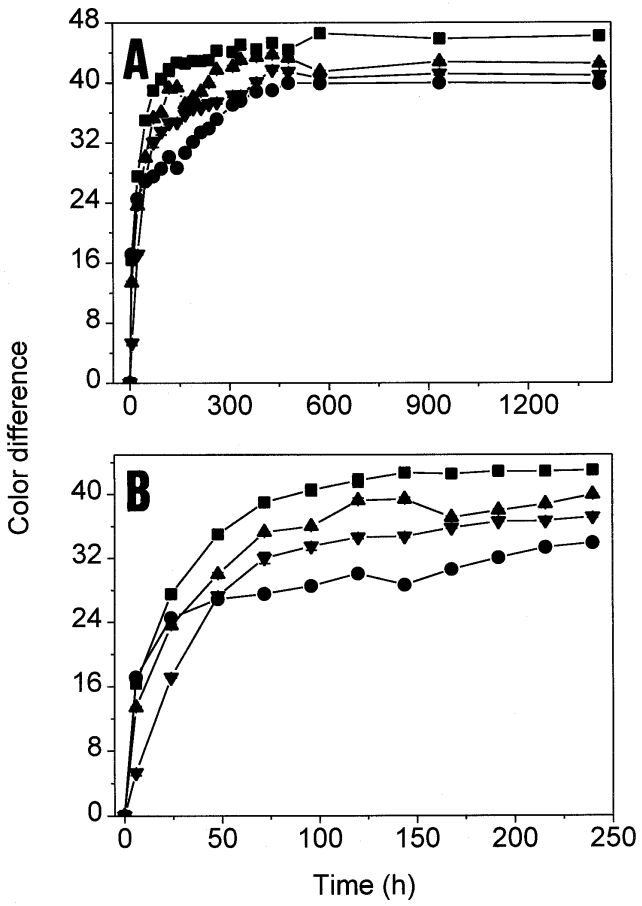

FIG. 5. Effect of the treatment on the changes produced in ΔE of a FA/BSA mixture. See Figure 2 for experimental details. Results represent the mean ± SD of three assays. The first 250 h of treatment are expanded in part B of the figure. See Figure 1 for abbreviations.

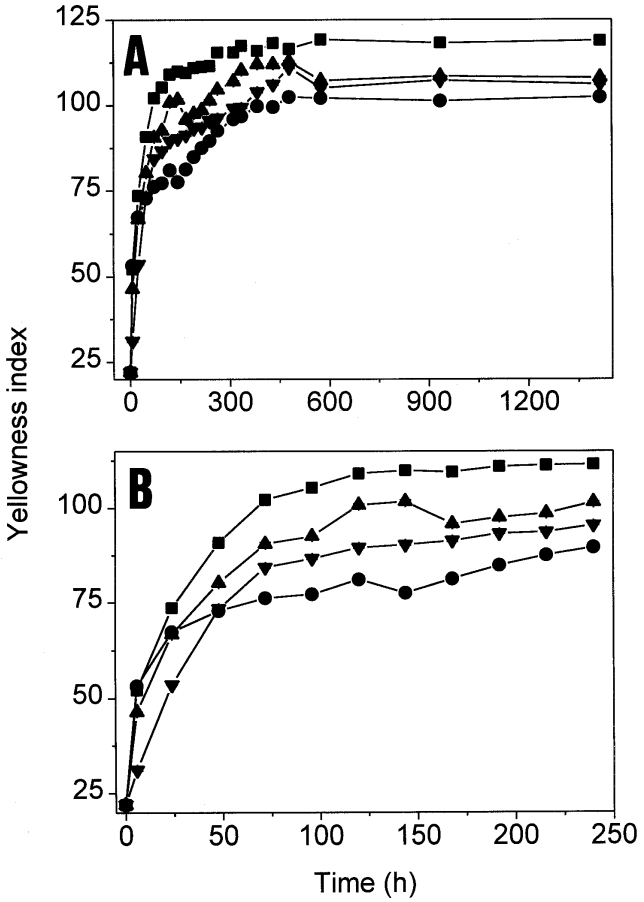

FIG. 6. Effect of the treatment on the changes produced in YI of a FA/BSA mixture. See Figure 2 for experimental details. Results represent the mean ± SD of three assays. The first 250 h of treatment are expanded in part B of the figure. See Figure 1 for abbreviations.

ΔE increased very slightly and, after 48 h, these samples exhibited the significantly lowest ΔE of all experiments.

Analogous results to those obtained for ΔE were also obtained for YI. Figure 6 shows the changes in YI produced with time for the four treatments. Analogously to ΔE, there were smaller differences among the four treatments for YI than for lipid peroxidation, and samples had significantly decreasing YI in the following order: experiment A > experiment B > experiment C. Samples in experiment D had a value of YI as high as samples in experiment A after the first 6 h, and then YI increased very slightly. After 48 h, samples in experiment D had the significantly lowest YI of all experiments.

DISCUSSION

Unlike oxidation of pure lipids, when lipid oxidation occurs in food products, other reactions can occur. Among all these reactions, the protein damage produced by lipid oxidation products has long been known, mainly because of their negative consequences on food proteins (25–27). However, recent studies from this laboratory have suggested a possible positive role for these reactions because OLAARP produced from oxidized lipid/protein reactions exhibited antioxidative activities and, therefore, their presence might increase food stability relative to oxidation (28,29). In addition, the OLAARP could be generated *in situ* and therefore would increase the oxidative stability of the food without the use of external additives.

The oxidation of an FA/BSA mixture under air (experiment A) initially produced lipid hydroperoxides, as indicated by the increase in the absorbance at 234 nm. These hydroperoxides decomposed to produce the secondary products of lipid oxidation, as could be determined by the absorbance at 270 nm or by TBARS. When samples were stored under nitrogen (experiment B), lipid oxidation was delayed, although it was not completely inhibited, and the final values of lipid oxidation products were lower than the values obtained for oxidation under air. Similar behavior was observed in the FA/BSA mixture when it was treated with BHT (experiment C). The synthetic phenol delayed lipid peroxidation, and samples treated with it exhibited significantly higher stabilities than samples incubated under nitrogen. However, the most stable samples were obtained when the FA/BSA mixture was incubated first under air for 6 h and then under nitrogen (experiment D). Thus, although the first 6 h produced a substantial lipid peroxidation that seemed to indicate that these samples were less stable than samples incubated under nitrogen or with BHT, this early lipid peroxidation later produced the most stable samples. Table 1 summarizes the results obtained in this study and arranges the significant efficiency of the four treatments as a function of the lipid peroxidation of the samples, evaluated by the TBARS assay, and the discoloring produced, as determined by using YI.

Although no determinations of OLAARP have been carried out in this study, previous research on these reactions suggests a possible explanation for these results. The presence of oxygen for the first 6 h should oxidize some polyunsaturated fatty acids, producing the corresponding hydroperoxides. This is in agreement with the increase in absorbance

TABLE 1
Efficiency of Treatments to Prevent Lipid Peroxidation and Discoloring in Fatty Acids/Bovine Serum Albumin (FA/BSA) Samples as a Function of Incubation Time[a]

Time (h)	Lipid peroxidation[b]	Color changes[c]
6	A ≅ D < B ≅ C	A ≅ D < B < C
24	A < D < B ≅ C	A < D < B < C
48	A < D < B < C	A < B < C < D
96	A < D ≅ B < C	A < B < C < D
168	A < B < D ≅ C	A < B < C < D
240	A < B < C < D	A < B < C < D

[a]FA/BSA samples were incubated under: A, air; B, nitrogen; C, air in the presence of 200 ppm of butylated hydroxytoluene; and D, air for 6 h and then nitrogen. Group differences were evaluated by the Student-Newman-Keuls test. Significance level is $P < 0.05$.
[b]Lipid peroxidation was evaluated by the thiobarbituric acid-reactive substances (TBARS) assay. Only significant efficiency of the treatments is shown. A higher efficiency was indicated by a significantly lower TBARS production.
[c]Color changes were evaluated by determination of the Yellowness Index (YI). Only significant efficiency of the treatments is shown. A higher efficiency was indicated by a significantly lower YI.

at 234 nm observed. However, when the air was replaced with nitrogen, lipid oxidation was no longer favored, as it occurs in the presence of air, and the absorbance at 234 nm did not show any new increase. The lipid hydroperoxides should react with reactive groups on neighbor proteins producing OLAARP (15,30–32) with antioxidative properties (28,33). The stability obtained with the treatment was sufficient so that, after 168 h, samples oxidized in air for 6 h had significantly less TBARS than samples incubated under nitrogen from the beginning. In addition, this treatment did not produce unsatisfactory discoloring in the samples, which exhibited the lowest ΔE and YI.

Lipid oxidation leading to rancidity is often the decisive factor for determining the useful storage life of food products, even when their fat content is low (34). Therefore, substantial effort has been undertaken for many years to reduce lipid peroxidation in order to extend the shelf life of food products. However, the above results are not in agreement with this postulate, and they suggest that a slight and controlled lipid oxidation might improve the stability of food products to oxidation. Additional studies are needed to establish the applicability of this new concept to the preservation of real foods and to confirm if slightly oxidized foods might have organoleptic properties that are acceptable for consumers.

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